

**SHORT-TERM EARLY LIFE STAGE
GROWTH TEST USING SACFRY AND
EARLY SWIM-UP STAGES OF RAINBOW
TROUT (*ONCORHYNCHUS MYKISS*)**

PROTOCOL

AUGUST 1995

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**Ministry of
Environment
and Energy**

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USING SACFRY AND EARLY SWIM-UP STAGES OF
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PROTOCOL**

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ABSTRACT

The protocol for this short-term, rainbow trout early life stage growth test has been developed to provide a simple, cost effective test with the least possible uncertainty to estimate the concentrations of toxicants that are likely to cause chronic toxic effects in the aquatic environment. The late sac fry to swim-up fry life stage is used as this is the shortest, most sensitive period which includes both uptake of toxicants and expression of effects. The fry are exposed in individual containers at $13.5 \pm 0.5^{\circ}\text{C}$, and at swim-up are fed equal volumes of a thick suspension of live 24 - 48 hour old brine shrimp nauplii for 5 days, (routine test), or 14 days using chemicals with low absorption rates. Growth is measured as the gain in wet weight, and sublethal test concentrations are used to provide greater sensitivity than tests using survival and growth as dual endpoints. Growth effects using this short-term protocol are as sensitive as longer ELS protocols for estimating the effect concentrations of metals, surfactants and organic chemicals in chronic exposures, (see Neville, 1995). The protocol can be used for monitoring possible chronic toxicity levels in non-acutely lethal effluents, receiving waters, leachates and elutriates, as well as providing data for the development of Federal and Provincial Water Quality Standards for individual chemicals. This method should be used under the supervision of personnel experienced in sublethal aquatic toxicity tests using early life stages of fish.

RÉSUMÉ

La méthode employée pour le test d'inhibition de la croissance effectué sur des truites arc-en-ciel du stade de l'alevin vésiculé avancé au surnageant se voulait simple et peu coûteuse, avec un facteur d'incertitude qui soit le moins élevé possible. Le but du test était d'évaluer les concentrations de produits toxiques susceptibles d'entraîner des effets toxiques chroniques en milieu aquatique. Nous avons étudié le stade de l'alevin vésiculé avancé au surnageant pour deux raisons : cette période est la plus courte qui comprenne et l'absorption de substances toxiques et l'expression des effets, et c'est la période où l'alevin est le plus vulnérable. Les alevins ont été exposés aux polluants toxiques dans des récipients séparés maintenus à une température de $13,5 \pm 0,5$ °C. Au stade du surnageant ils ont été nourris de volumes égaux d'une suspension épaisse de nauplius d'artemia vivants et vieux de 24 à 48 heures et ce, pendant 5 jours pour le test de routine et pendant 14 jours pour les substances chimiques ayant un faible taux d'absorption. La croissance est mesurée en fonction de la prise de poids frais. Les concentrations sublétales utilisées dans l'essai fournissent une plus grande sensibilité que les essais où les taux de survie et de croissance constituent la combinaison des deux effets significatifs. Les effets sur la croissance mesurés à l'aide de cette méthode à court terme sont aussi sensibles que ceux qui sont obtenus selon des méthodes de plus longue haleine faisant appel aux premiers stades larvaires pour mesurer les concentrations de métaux, d'agents tensioactifs et de substances chimiques organiques entraînant des effets lorsqu'il y a exposition chronique (Neville, 1995). La méthode peut s'avérer utile dans la surveillance des niveaux de toxicité chronique des effluents à létalité non aiguë, des eaux réceptrices, des lixiviats et des éluats, et dans la compilation des données nécessaires à l'établissement de normes de qualité provinciales et fédérales pour différentes substances chimiques. La méthode doit être utilisée sous la surveillance de personnes qui ont l'habitude des essais de toxicité sublétale en milieu aquatique effectués sur des poissons aux premiers stades larvaires.

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LIST OF ABBREVIATIONS

°C.....	degree(s) Celsius
cm.....	centimetre(s)
c.v.	coefficient of variation
d.....	day(s)
DCA.....	dichloroaniline
ELS.....	early life stage
fct.....	footcandle, a unit of illumination based on units per square foot
h.....	hour(s)
IC _x	inhibiting concentrations causing the % differences in wet weight gain of the LOEC and NOEC from the control, in a specified test
L.....	litre(s)
LOEC.....	lowest-observed-effect concentration
log K _{ow}	logarithm of the octanol-water partition coefficient
lx.....	lux, a unit of illumination based on units per square metre. One lux = 0.0929 foot candles, (fct), one foot candle = 10.76 lux.
mg.....	milligram(s)
mL.....	millilitre(s)
mm.....	millimetre(s)
MSD.....	minimum significant difference
n.....	number of observations
NOEC.....	no-observed-effect concentration
s.d.	standard deviation
µg.....	microgram(s) (1 µg = 1/1,000 of a mg)
µg/L.....	micrograms per litre

LIST OF SYMBOLS

$^{\circ}$	degrees (geometric)
%	percent
"	inch(es)
$\approx$	approximately
$\bar{x}$	mean
$\pm$	plus or minus
$<$	less than
$>$	greater than
$\leq$	less than or equal to
$\geq$	greater than or equal to

INTRODUCTION

A sublethal, subchronic early life stage test applicable to coldwater fish is needed for regulatory purposes for the protection of the aquatic environment, and for developing multimedia criteria. As such, the test must be cost effective yet sensitive to a wide range of chemicals, and its interpretation must be clear. A national protocol published by Environment Canada in 1992 includes three tests. One is a 7 day, freshly fertilised egg mortality test that is cost effective but not necessarily sensitive to sub-chronic or chronic levels of toxicity. The second is a two month teratogenicity test and the third is a standard three month ELS test with mortality, teratogenicity and growth as endpoints. This test is sensitive to a wide range of chemicals, but, like the two month test, is not cost effective. A short-term growth test which would satisfy all three requirements is not included. Growth has been shown to be the most sensitive endpoint in three month, early life stage tests using salmonids, (Hodson et al, 1991), but its variability in test protocols developed until now has been a deterrent in using this endpoint in short-term, salmonid early life stage tests. The protocol described here is a cost effective, short-term ELS growth test, using the shortest combination of most sensitive life stages in rainbow trout for the uptake of toxicants and expression of effects. Growth is measured as the wet weight gain of individually exposed fish to avoid the variability found in other growth tests.

Preliminary stages in the development of this protocol have been cited as one of the procedures used in developing the Environmental Protection Series, Biological

Test Method: Toxicity Tests Using Early Life Stages of Salmonid Fish (Rainbow Trout, Coho Salmon, or Atlantic Salmon), (Environment Canada, Report EPS 1/RM/28, December, 1992). Some details of the test methodology and related information have been changed in this protocol. It is intended for use with EPS 1/RM/28, in particular, growth related procedures in the List of Abbreviations, (pp. xiii-xiv); Terminology, (pp xv - xxiii); and the sections Test Organisms - (2.2, Source); Test System - (3.1, Facilities and Materials, 3.4, Control/Dilution Water); Universal Test Procedures - (4.1, Preparing Test Solutions, (except where noted below), 4.3.9, Reference Toxicant, 4.7, Legal Considerations); Specific Procedures for Testing Chemicals - (5.2, Properties, Labelling, and Storage of Samples, 5.3, Preparing Test Solutions, paragraph 1, 5.4, Control/Dilution Water, and 5.5 for the stability of exposure solutions); Section 6, Specific Procedures for Testing Samples of Effluent, Elutriate, and Leachate; Section 7, Specific Procedures for Testing Receiving-water Samples; and Section 8, Reporting Requirements. A few changes to these sections have been noted where appropriate in this protocol.

TERMINOLOGY DIFFERENCES FROM EPS REPORT 1/RM/28

Growth Measured as gain in wet weight.

MSD Minimum significant difference from the control, calculated in the Dunnett's and Bonferroni T tests.

Subchronic Term used in this protocol for exposure periods from 12 to 21 days.

TEST ORGANISM AND LIFESTAGE Rainbow trout, late sacfry to feeding swim-up fry.

Source, culture and acclimation of the sacfry Seven hundred late eyed rainbow trout eggs should be obtained from a certified disease free trout farm, and transported to the laboratory using food grade polyethylene bags with 100% air saturated water and a large head space, kept in coolers maintained at the same temperature as the trout farm culturing facilities. In the laboratory, they should be incubated in hatching trays at the same temperature until hatching is complete, with approximately 250 - 350 eggs per tray, using filtered, aerated, flowing water, with additional treatment as required depending on the water supply, (eg. if a chlorinated water supply has to be used, dechlorination and UV treatment to ensure that total chlorine is $\leq 15 \mu\text{g/L}$). The eggs should be examined twice daily and any dead or fungussed eggs should be discarded. After hatching, the temperature should be increased by 1°C every 2d to 12°C and maintained at that temperature until 9 - 11d post hatch. The temperature, and number of abnormal and fungussed eggs discarded, should be recorded daily.

After hatching commences, the sacfry should be transferred to labelled hatching trays at the beginning and end of each day, to minimize the age difference between individual sacfry in any tray. They should be transferred with an 8-10 mm wide bore pipette and rubber bulb, using only sufficient pressure to allow the sacfry and water to be sucked into the first 8 to 10 cm of the pipette. The pipette should be held at an angle of $\approx 20-25^{\circ}$ whilst sucking up the sacfry and $5-10^{\circ}$ whilst transferring them. The trays should be labelled with the date, time, and approximate number of sacfry. The first and last groups of sacfry to hatch must be transferred but should not be used in the test as these usually include most of the abnormal sacfry. If more than 10% of the sacfry are abnormal

in any other tray, the sac fry in that tray should also be excluded from the test. Any other fungussed, malformed or abnormally swimming sac fry should be recorded and removed twice daily. Incubation of the healthy sac fry should continue for 9-11d post hatch at $12 \pm 1^{\circ}\text{C}$ before test procedures are initiated.

TEST SYSTEM

The sac fry are exposed to the control and five test concentrations in a static, renewal system under low fluorescent light conditions (≈ 30 lux), with a 16h light/8h dark photoperiod, at $13.5 \pm 0.5^{\circ}\text{C}$ for 12 - 21 days, (depending on the test substance, see p. 22). Twelve sac fry are used per concentration. They are exposed in individual chambers held in three replicate, covered tanks subdivided into four equal, separate, sections (see Figure 1). Since the individual fish are weighed and exposed separately, each fish is a replicate.

Exposure system construction materials Borosilicate glass or teflon are preferred construction materials for the exposure tanks and chambers, with teflon being easier to use because of its lighter weight. However, polycarbonate is a clear, colourless plastic that is cheaper than teflon, and can withstand the concentrations of solvents required for cleaning the exposure chambers and tanks after they have been exposed to the sublethal chemical concentrations used in this growth test. Polycarbonate is available in 1/8" sheets that can be used to construct the tanks and chambers. Methylene chloride, a plastic solvent, is used as the glue. Nylon mesh bases for the individual chambers are glued to the polycarbonate sides with a thick paste formed by dissolving nylon mesh shavings in the methylene chloride.

Exposure system The individual exposure chambers and the separate sections of the tanks containing them must be tall and narrow, with a volume to base area ratio of at least 13 cm for the inner chambers and 10 cm for each of the tank sections. This design allows sufficient swimming room at the swim-up fry stage, easy estimation of the approximate amount of food consumption of each test group compared to the controls, (excess food settles into a small area), and restricts the loss of volatile chemicals. Four individual chambers are held in each of eighteen exposure tanks. Two sets of the exposure tanks are required.

During the development of this protocol the individual exposure chambers were 2 1/2" x 2 1/4" x 5 1/8" deep, (outside measurement), with nylon monofilament mesh bases (Nitex, ASTM3-14-1410; thread diameter = .0236", 12.6 holes per inch). The exposure tanks were 5 7/8" x 5 1/4" x 5 1/4" deep, (outside measurement), divided into four equal, separate sections, each containing 400 ml exposure solution, (see Figure 1). Two strips of polycarbonate placed near the top of opposite sides of the tanks were used for ease of handling. Individual polycarbonate covers (5 1/4" x 5 7/8") were placed over each of the exposure tanks to avoid contamination of the exposure solutions or significant loss of any volatile chemicals¹.

The tanks were incubated at $13.5 \pm 0.5^{\circ}\text{C}$ in a large, opaque temperature controlled water bath with an opaque plexiglass cover. A plexiglass stabilizer tray, with holes slightly larger than the exposure tanks, were used to hold them in place, allowing the water to circulate easily between tanks.

¹A small headspace is required to prevent the swim-up fry from swimming over the edge of the individual chamber.

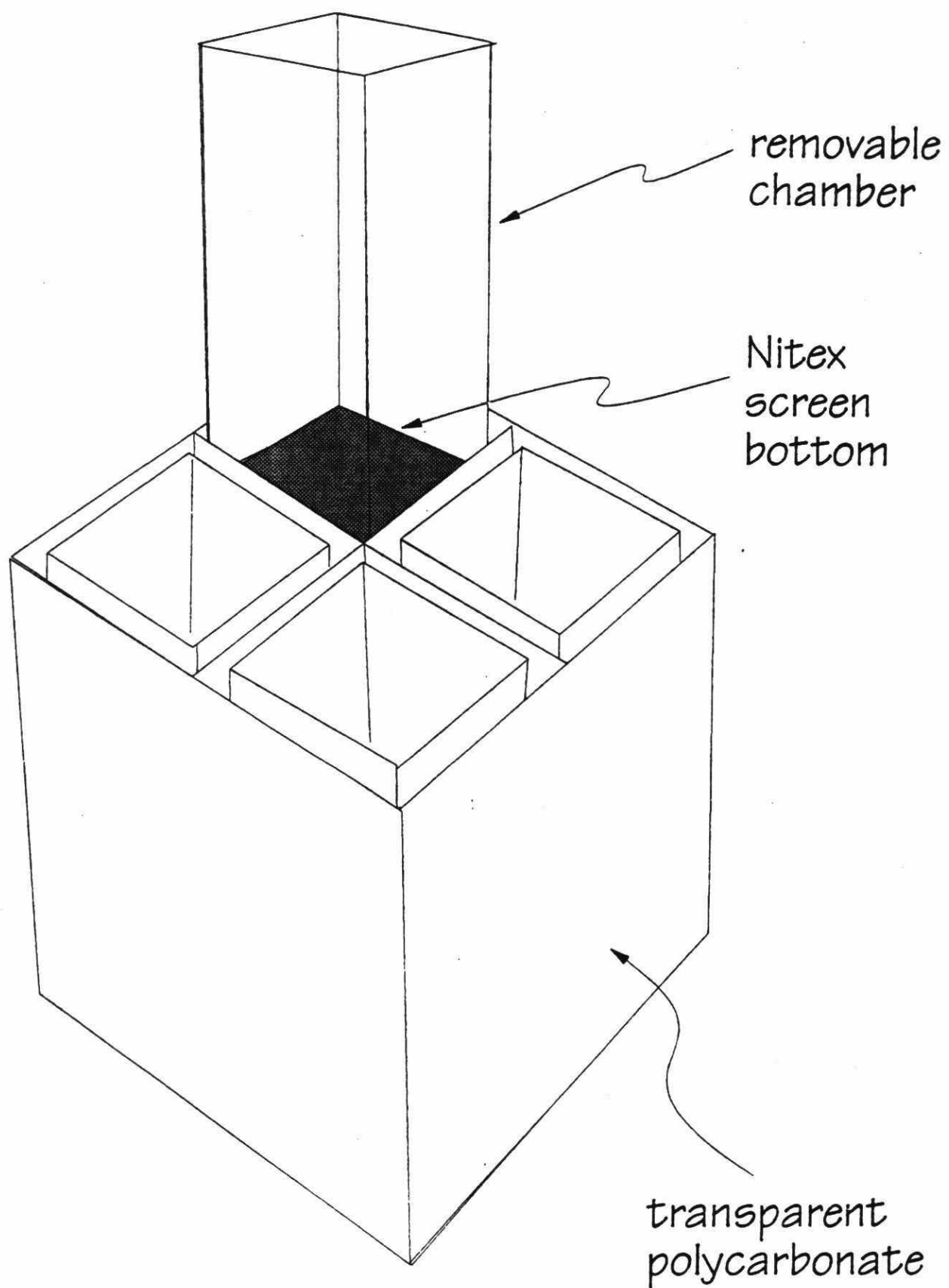


Figure 1. Diagram of Exposure Apparatus

(Polycarbonate cover, and 2 polycarbonate strips used for ease of handling, not shown)

Lighting Subdued lighting should be used throughout the test. Light intensity at the water surface should be 20 - 30 lux, fluorescent light, with a photoperiod of $\approx$ 16h light/8h dark, and preferably, a 15 - 30 minute transition period.

Temperature $13.5 \pm 0.5^{\circ}\text{C}$.

GENERAL TEST PROCEDURES

The test system described above must be used with these general test procedures when using the growth test with specific chemicals, effluents, receiving waters, elutriates and leachates. Any specific procedures for each type of test are described in later sections. A summary of the recommended conditions and procedures is shown in Table 1.

Preparing test solutions Only non-lethal test concentrations should be used in this growth test. Normally, for all tests except receiving waters, a series of five double dilutions should be used, with concentrations ranging from the low, not expected to be significantly different from the controls, to one close to, but below, the incipient 12 day mortality level for fry. If unknown prior to the test, this should be determined for chemicals by a range finding test, using 12 day post hatch sac fry in a 12 day static incipient mortality test with a 24h solution renewal period, without aeration. Feeding, using the same methodology as in the growth test (see below), should start on the 7th day and continue for 5 days; oxygen concentrations should be monitored daily during this stage and the exposure solutions renewed more frequently if required. For non-acutely

Table 1Test Conditions and Procedures¹.

Test type	- covered static-renewal
Species, life-stage	- rainbow trout, late sacfry to 5d post swim-up
Start of test	- at age 10-13d post hatch, 24h after weighing the sacfry
End of test	- 18h after feeding the swim-up fry for 5d, at age 22-25d (feeding may be continued up to 14d, extending the total exposure period from 12d up to $\approx$ 21d if required for some chemicals with low absorption rates or with narcotic properties eg. 3,4-DCA, see p. 22)
Control/dilution water	- ground, surface, reconstituted, or if necessary, dechlorinated municipal water; upstream water to assess toxic impact at a specific location
Test apparatus	- individual polycarbonate exposure chambers with screened bases, exposed in covered polycarbonate tanks subdivided into 4 equal sections, 3 tanks per concentration
Number of organisms	- 72, (12 per control and 5 test concentrations)
Number of replicates	- 12 per concentration, (each fish is a replicate)
Temperature	- $13.5 \pm 0.5^{\circ}\text{C}$
Oxygen/aeration	- aerate control/dilution water before use to ensure 90 to 100% dissolved O_2 saturation, (any supersaturation must be reduced to 100%). No aeration during test.
pH	- in range of 6.5 to 8.5. If toxicant is pH dependant, stock solution must be adjusted appropriately before preparation of exposure solutions
Lighting	- 30-40 lux, fluorescent, 16h L/8h D
Feeding	- at swim-up, (age $\approx$ 18 days), after 6 days exposure of the sacfry. 1 drop of a concentrated suspension of 24-48h old brine shrimp nauplii per fish, one feed mid-afternoon on the first day, three feeds per

(Table 1, contd.)

day until the final day, no feeding during the final 18 hours of exposure.

Observations

- initial wet weight; any abnormal behaviour or appearance compared to the controls during exposure period; age at onset of feeding or faecal production and estimated amount of food eaten compared to controls every 24 hours during feeding stage (noted in used exposure solution after solution renewal); final wet weight; width of gap (0, <1, 1-2, or >2 mm) in epithelium over yolk sac if adsorption delayed; any abnormal appearance compared to control at end of test; dry weight if required for tissue toxicant analysis.

Measurements

- temperature, continuously in waterbath, daily in replicate tanks; oxygen saturation, pH, and conductivity daily in pre-exposure control and test solutions and post 24 hour replicate tank exposure solutions; oxygen saturation in the individual chambers after approximately 18 hours exposure during the 5 days feeding at the swim-up fry stage. Routine water quality parameters, heavy metal concentrations, dissolved organic carbon, chlorine (if dechlorinated water is used for control and dilution water), and toxicant exposure concentrations when individual chemicals are being tested, in pre- and post-24 hour control and test exposure solutions on the second and eleventh days of the test, (ie. once during the sac fry stage and once during feeding of the swim-up fry). Free ammonia and nitrite concentrations in the eleventh day post-24 hour control and test group samples.

Endpoints

- wet weight gain NOEC/LOEC, (see p. 20), and MSD, (minimum significant difference from controls). The NOEC and LOEC can be used as ICx. Maximum width of exposed yolk sac surface, when yolk sac absorption delayed.

Reference toxicant

- copper, or zinc, and/or phenol. The complete 12 day growth test should be used while the test is being set up in the laboratory, and at least twice each year thereafter. During

(Table 1. contd.)

	each of the intervening months a range-finding test, using three $\geq$ 10 day old sacfry and 3 swim-up fry in a 96 hour static-renewal acute test, without aeration, may be substituted for the growth test whilst the source of eggs, culturing, and test exposure conditions, including personnel, remain constant.
Solvents	- solvents are not required at the sub-lethal concentrations used in this growth test.
Test validity	- invalid if one of the following occurs: exogenous feeding of controls not established after 1 day of feeding; >10% mortality in the controls; <45% wet weight gain in the controls; CV of initial weights of all test fish >15%; CV of control fish final wet weights >10%; CV of control wet weight gain >20%; temperature difference among exposure tanks >1°C; oxygen saturation in any exposure tank <55% or >103%.

¹ Format as in Table 3, Environment Canada Report EPS 1/RM/28, December 1992

lethal effluents, leachates and elutriates the dilution range should be estimated from a 96h static incipient mortality test with a 24h solution renewal period, using 3 late sacfry and 3 early swim-up fry in each concentration, without aeration or feeding. The highest test concentration used in the 12 day growth test should be ≤ 0.5 of the 96h incipient mortality concentration. The subsequent field samples must be tested for 24h before being used in the growth test, using an appropriate range determined by the first field sample, adjusted, if required, to account for significant differences in flow conditions at the time of sampling², then run concurrently with the growth test. For regulatory purposes, the highest concentration may be used with a control, in single concentration tests used as pass/fail tests.

Fresh test solutions must be made daily from stock chemical solutions, or from well mixed, undiluted samples of non-acutely-lethal effluents, leachates, or elutriates. Receiving water samples are used without dilution. The pH of stock solutions (or substock solutions) should be appropriately adjusted before the exposure solutions are prepared if it is known that pH dependent toxicants are present. There should be no pH adjustment during the test.

Stability of stock and exposure solutions See Environment Canada EPS Report, 1/RM/28, section 5.5.

Control/dilution water See Environment Canada EPS Report, 1/RM/28 section 3.4,

²In chlorinated effluents from sewage treatment plants the chlorine concentration must be measured before setting up the 96h incipient mortality test to ensure that dilution of each effluent sample will be sufficient to avoid acute mortality in the highest concentration(s) due to chlorine.

pp. 12-13, and Figure 2, p. 14. with the following changes:-

Dissolved carbon dioxide	<5.0 mg/L
Dissolved nitrogen	100-103% of air saturation level.
Dissolved oxygen	90-100% of air saturation level.

The control and dilution water must be aerated at $13.5 \pm 0.5^{\circ}\text{C}$ before use to achieve 100% air saturation in the controls and optimal air saturation in all test concentrations at the start of the test. There must be no supersaturation.³ No aeration must be used during the test. Oxygen consumption levels during the life stages used in this test are less than those of juvenile fish, since sacfry activity is low, and swimming activity of the swim-up fry during feeding is kept relatively low by the shape of the exposure chambers in order to maximise growth. However, air saturation levels should not be <55% by the end of each 24h exposure period.

Weighing procedure - Pre-test weights Eighty-two sacfry, aged $10 \pm 1\text{d}$ post hatch, with weights ranging within $\pm 10\%$ of the median value, are initially weighed and kept overnight in individual numbered chambers immersed in control water at $13.5 \pm 0.5^{\circ}\text{C}$, during an 18-24h recovery period. (The preferred weight range is 100 - 120 mg, but this will vary with the age of the spawning female, first spawning females generally producing smaller eggs). Ten of the weighed fish are used as spares in case any fish show stress, (very dark colouration), after the recovery period. (With experience, this rarely occurs in more than two or three fish).

³Supersaturation in the control and dilution water will decrease growth to the greatest extent in the controls, and increase growth in toxicants with a high oxygen demand.

Hatching tray supply water must be used in temporary holding tanks, beakers, and basins containing individual exposure chambers held in waterbaths at $12.5 \pm 0.5^{\circ}\text{C}$ throughout the weighing procedures, and must be renewed frequently to ensure that the oxygen saturation as well as the temperature remains as stable as possible, to minimise stress to the sacfry. Sunlight, or bright artificial light must be avoided in the weighing room.

The hatching tray with the largest number of healthy sacfry is used to initiate the test. Approximately 20 sacfry should be removed by netting to a rectangular glass holding tank ($\approx 8" \times 2 \frac{1}{2}" \times 3 \frac{1}{4}"$) containing ≈ 750 mL water at a depth of $\approx 2"$, and transferred to a waterbath in the weighing room. Any sacfry with a notable difference in size of the larva and/or yolk sac from the majority of fish should be discarded. (This should not occur in more than 5% of the fish).

The weighing vessel should be a small, round container of ≈ 80 mL capacity, made of clear, light weight, food grade plastic, with a loose fitting, air tight lid of similar material. It should be half-filled with fresh water kept in a large beaker in the waterbath. The outside is dried if necessary, then it is tared to zero with the lid on, using a Mettler AT 250 or similar balance, accurate to $100 \mu\text{g}$, (preferably with automatically controlled doors).

Using a wide bore pipette, (8-10mm diameter), held at an angle of $\approx 25^{\circ}$, a sacfry is transferred from the holding tank with ≈ 2 mL water to a small, nylon mesh cage, (4 x 2.5 x 2.5 cm, Nitex sceening, ASTM3-14-1410), and covered with a nylon mesh lid. The outside of the cage and surface epithelium of the sacfry are thoroughly but gently blotted (through the nylon mesh) with dry extra low-lint

wipers before the sacfry is tipped into the weighing vessel without displacing any water. The lid is quickly replaced, the balance door closed, and the weight recorded to the nearest 0.1 mg as soon as it first stabilises, (usually within 5 seconds of the door closing). The sacfry is then tipped from the weighing vessel into a numbered, individual exposure chamber in a temperature controlled tank containing hatching tank water, temporarily held in a waterbath in the weighing room. The whole procedure for each fish is completed within 1 minute. The blotting procedure takes less than 10 seconds.

The acceptable 20 mg weight range, preferably somewhere between 90 and 130 mg, can be determined after the first 20 - 30 fish have been weighed. (It is possible to use 9-11 day old sacfry with initial weights as low as 65 mg, but weight gains will be lower than those of larger fish, increasing the risk of errors reducing the test sensitivity, and description of the fish compared to the controls may be difficult.) Fish outside the acceptable weight range should be discarded. Capturing, transferring, and weighing the sacfry can be done smoothly and gently using the equipment and procedure as described, causing minimal stress to the fish remaining in the holding tank as well as to the captured fish.

Weighing is continued, using fresh water in the weighing vessel for each sacfry. After all suitable fish in the glass holding tank have been weighed, the temperature controlled tank containing the exposure chambers is transferred to a large waterbath in the bioassay room. The balance is allowed to automatically recalibrate if required whilst 20 more sacfry and fresh water supplies are collected from the hatching room. The whole procedure is repeated until 82 suitable test and spare sacfry have been weighed. If necessary, the hatching

tray with sac fry nearest in age to the first group is also used. An 18 to 24h recovery period is allowed, then any stressed sac fry in the first 72 numbered exposure chambers are replaced with unstressed spare fish.

Use of the nylon mesh cage rather than direct blotting reduces the likelihood of any sac fry being stressed, but does include $\approx$ 2 to 5 mg water still adhering to the body surface. The initial weights should be adjusted by deducting the average amount of water carried over, calculated for each test from the difference in weights of six sac fry from the same hatching period, each measured first by using the mesh cage for drying, then immediately again after directly blotting the body surface. These fish are then discarded.

Test initiation For each of the control and five test exposure concentrations, 400 mL of the exposure solution at 13.5°C are added to the four separate compartments of three appropriately labelled replicate exposure tanks. The different concentrations should be distributed randomly in the test exposure waterbath. However, the replicates of each concentration should be assigned to different areas within the tank to ensure uniform exposure to any slight differences in light or disturbance during the test procedure. Seventy-two fish in the numbered individual exposure chambers are then transferred to the exposure tanks. The fish are distributed randomly except for the last few which should be distributed non-randomly to minimize the weight range among exposure tanks, and if fish from a second hatching tray have had to be weighed, all of these fish must be distributed first. Labelled polycarbonate covers are then placed on each tank. The fish number, initial weight, and position in each replicate tank are recorded.

Solution renewal The fish should be transferred to fresh exposure solutions every 24h at mid-afternoon during the sacfry stage, and in the early morning as well as mid-afternoon during feeding of the swim-up fry, if the oxygen saturation levels in any group are less than 65% after 18h exposure. (This rarely occurs). For volatile chemicals, the pre- and post-24h exposure concentrations must be measured in a preliminary test without fish to determine the appropriate renewal period. A second set of replicate exposure tanks is used for solution renewal in order to minimise disturbance to the fish. Each individual exposure chamber is raised slowly from the used exposure solution and immediately transferred to the appropriate tank containing fresh exposure solution at $13.5 \pm 0.5^{\circ}\text{C}$. Any fish showing grossly abnormal appearance or behaviour from the controls can be noted at that time.

During the swim-up stage, after feeding initiation, the waste matter left in the used exposure tanks is allowed to settle, then examined briefly against a white background and with the aid of a light box, to rank the amount of food and faecal threads present in each group compared to the controls, (eg. 0, + (control rank), ++, +++). The faecal threads are usually red and easily seen. These records are used to determine the age at which exogenous feeding began and/or faecal threads were first produced, the relative amount of daily food consumption compared to other groups, and whether food consumption was maintained until the end of the test, or reduced after the first few days. (The latter may occur in some groups when the fish are exposed to chemicals with narcotic properties, eg. 3,4-DCA; see Neville, 1995, for discussion of data interpretation).

Feeding

After 6d exposure, at age 17-18d when the yolk sac is mostly absorbed but still visible, exogenous feeding is initiated. The amount of food given must be slightly in excess of control fish satiation; (too much excess food may reduce oxygen saturation levels below the acceptable range by the end of the 24h exposure period).

On the first day of feeding, one concentrated drop (≈ 5000 nauplii) of 24-48h old Artemia Platinum Grade brine shrimp, (Bio-Marine Brand Artemia cysts, Hawthorne, California), washed in a separating funnel with hatching tank water, is given to each fish in all groups, in the afternoon after solution renewal. To ensure that a similar amount of food is given to all fish, the washed brine shrimp are allowed to settle in the separating funnel to a thick suspension approximately 2 mL in volume. This is collected in a small beaker, (avoiding inclusion of egg shells), swirled quickly to maintain an even suspension and immediately taken up into an eye dropper, (inside measurement of tip = 2mm), then 1 drop is added to each individual exposure chamber whilst holding the eye dropper vertically to prevent settling out of the nauplii or air intake. A Pasteur pipette is not suitable for this procedure). The last drop in the eye dropper is not used. The beaker is reswirled each time the eyedropper is refilled. (An automatic drop dispenser may be used instead of an eye dropper as long as the dispenser is gently swirled after every four drops are dispensed, to ensure that the brine shrimp are evenly suspended and distributed to all fish).

Three feeds are given on the subsequent 4-5 days in the 12d test, in the early

morning (after solution renewal if required), at mid-day, and again at approximately 3:00 pm after the mid-afternoon solution renewal. (The nauplii survive for at least 4 hours in the sublethal exposure concentrations used in this test, and they do not bioaccumulate the toxicants). No food is given on the final day of the test. Feeding is extended up to 14 days in a 21d test, (see p. 22).

POST EXPOSURE PROCEDURES

Final weights Starting with one of the control tanks but then in random order, the exposure tanks are removed individually to a waterbath in the weighing room and the individual post exposure weights are measured by tipping the fry from its exposure chamber onto 1 ply, extra low-lint wipers, gently blotting it dry, then weighing it on tared weighing paper. (Weighing is completed in 5 seconds).

Observations After weighing each fish, the severity of any delay in yolk sac absorption is assessed by measuring the maximum width of the gap in the epithelial layer covering the yolk sac, (0mm = no delay, <1 mm = slight, 1-2 mm = moderate, >2mm = severe), and any visible abnormalities compared to the controls are recorded, including colour, swollen and/or haemorrhaged body tissues, exophthalmia, and any sign of injury.

Individual wet weight gains are calculated, and the group mean wet weight gain and standard error are plotted against the measured concentration⁴ of individual

⁴Neither the data nor the exposure concentrations should be transformed, using the sublethal concentration range required for this test.

chemicals, or % concentration of effluents, leachates and elutriates, to produce the dose/growth response curve. If edema is suspected in the highest concentration group(s), it should be verified by the shape of the dose/response curve (which will appear to reach an asymptote in edematous groups), and, if available, the amount of feeding compared to lower concentration groups. The dose/response curve should not reach an asymptote using wet weight gain as the growth parameter if the concentrations are below incipient mortality levels for the fry stage, and the fish are feeding exogenously without severe reductions compared to the controls.

Reference toxicants Copper, or zinc, and/or phenol must be used in this 12 day growth test when the test is first set up in the laboratory, and at least twice each year after the test has been successfully established. During each of the intervening months, a range-finding test using three $\geq$ 10 day old sacfry and three swim-up fry in a 96h static-renewal acute test, without aeration, may be substituted for the growth test whilst the source of eggs, culturing, and test exposure conditions, including personnel, remain constant. (See Environment Canada EPS Report, 1/RM/28, section 4.3.9, pp. 29-30 for detailed use of reference toxicants).

Routine chemical analyses Temperature must be recorded continuously in the waterbath containing the exposure tanks. The pre-exposure temperature, pH, conductivity, and oxygen concentrations must be measured daily in each concentration (including the control) after preparation of the exposure solutions. Oxygen concentrations in individual chambers must be measured at the end of each 24h (sacfry) or 18h and 24h (swim-up fry during feeding) exposure

period. Chlorine, if dechlorinated water used, test exposure concentrations (in specific chemical tests) or concentrations of known major toxicants in field samples, and total heavy metals (copper, nickel, lead, zinc, iron, cadmium and chromium), dissolved organic carbon, pH, hardness, and alkalinity, must be measured pre-exposure (in each concentration) and post-exposure (in composite replicate samples of each concentration) of the same 24h period on the second and next to last days of exposure, (ie. once during the sacfry stage and once during feeding of the swim-up fry). Total nitrogen, total ammonia, nitrate and nitrite concentrations must also be measured in the post 24h exposure samples. Free ammonia concentrations are calculated from the total ammonia concentrations, pH, and temperature, (Emerson et al., 1975).

Statistical analysis Mean individual gain in wet weight is used to calculate the significance of growth differences between the test groups and the control, using appropriate hypothesis tests, and the LOEC and NOEC as measures of ICx. Linear regression analysis is usually not appropriate in this sublethal growth test, since the concentration range used is relatively small compared to growth and mortality tests, and a normal, small, non-linear (non-hormetic) response is frequently observed in concentrations close to the LOEC; (see Neville, 1995, for discussion of statistical analysis).

The Toxstat 3.3 statistical program (Gulley et al., 1991) should be used for data analysis, including initial tests for normal distribution and homogeneity of variance. The data should be analysed at a 5% level of significance, without transformation, using control < treatment as the null hypothesis. "Outliers" must not be eliminated unless an obvious non toxicant related abnormality was

noted in that fish, (eg. injured or deformed mouth or gills), or edema (swollen body tissues) had been identified in a fish causing its "wet weight gain" to be invalid as growth data. In tests of individual chemicals, effluents, leachates or elutriates, Dunnett's Procedure (or Bonferroni's T-test if any mortalities have occurred) is used. These statistical procedures also calculate the minimum significant difference from the controls, (MSD), for each test. For receiving waters, Tukey,s test should be used.

Each individually exposed fish is regarded as a replicate as with this protocol there are normally no significant differences among the three tank replicates of each concentration group. This can be verified using Tukey,s test. Should a significant difference occur between any two replicates of a group and the third replicate, eight individual fish replicates can be used instead of twelve for that concentration.

Any group showing a severe reduction in feeding without a reduced wet weight gain compared to the next lower concentration group, indicating edema, (identified by an apparent "asymptote" in the dose/growth response curve) must not be included in the growth analysis. (Edema may occur occasionally in these sublethal tests if the highest concentrations used are too close to the incipient mortality level.) Dry weight should not be used to determine growth in the developmental stages used in this short-term test, unless the feeding period is extended, (see Neville, 1995). However, measuring the final dry weight will reduce the cost effectiveness of this test without increasing the sensitivity.

Validity of test The following limits assume all recommended procedures and

conditions were followed (see Environment Canada EPS Report, 1/RM/28, p.34, note 34).

Temperature should be $13.5 \pm 0.5^{\circ}\text{C}$; variation of daily temperature and among test chamber temperatures must be $\leq 1^{\circ}\text{C}$. Oxygen concentrations must be within the limit for sacfry and swim-up fry under the conditions of this test, ($\approx 10.5 - 5.8$ mg/L; ie. 100% to 55% of air saturation level at $13.5 \pm 0.5^{\circ}\text{C}$; duration of exposure to less than 6.3 mg/L should be less than 4h). pH should be between 6.5 and 8.5, preferably 7.5 to 8.0. Exogenous feeding of controls should be established within one day of feeding. There should be no control mortality; test invalid if $>10\%$ control mortality. Initial weight coefficient of variation of all individual fish used per test should be $\leq 10\%$. Control fish wet weight gain should be $\geq 50 \pm 0.5\%$, and coefficient of variation should be $\leq 20\%$.

SPECIFIC PROCEDURES FOR TESTING INDIVIDUAL CHEMICALS

The normal 12 day exposure period must be extended to 21 days when testing chemicals with low absorption rates, (eg. organic chemicals with $\log K_{ow} \geq 4$). The sacfry must be initially weighed at age 9d post hatch, and the test must begin at age 10d. Feeding of the swim-up fry should continue for 14 days. The exposure period must also be extended if any of the test groups appear to establish normal exogenous feeding and food consumption within 2 days of the controls, but subsequently show(s) reduction in the estimated amount of food consumption, as may occur in chemicals with narcotic properties, eg. 3,4-DCA. (See Neville, 1995). The test must be extended until the food consumption either ceases, or is shown to have resumed former levels, or for up to 21 days exposure.

SPECIFIC PROCEDURES FOR TESTING EFFLUENTS, LEACHATES, AND ELUTRIATES

The sample volume required per day for this growth test is 12 litres, assuming that the toxicity level of the full strength sample approximates the incipient acute mortality level to rainbow trout sac fry and swim-up fry, and a five concentration series of double dilutions will be tested. This volume includes the requirements for a preliminary range finding test and samples for chemical analyses. Single concentration pass/fail tests require 2 litre samples.

Tests with field samples must begin with sac fry at age 10d post hatch, (initially weighed at age 9d). The exposure period must be extended to 21 days for leachates and elutriates. Similarly, the exposure period must be extended to 21 days for effluents which contain, or may contain chemicals with low absorption rates, (eg. organic chemicals with $\log K_{ow} \geq 4$). For any other effluents in which any of the test groups appear to establish normal exogenous feeding and food consumption within 2 days of the controls, but subsequently show(s) reduction in the estimated amount of food consumption, the exposure period must be extended until the food consumption either ceases or is shown to have resumed earlier levels, or up to 21 days.

See Environment Canada EPS Report, 1/RM/28, Section 6, pp.41-45, for information on use of samples containing suspended solids; sample collection, labelling, transport and storage,⁵; preparing test solutions,⁶; control/dilution water;

⁵Aliquots of the sample must be adjusted from the transport or storage temperature to 13.5°C before testing. Supersaturated samples should be well aerated for ≈ 5 minutes before use. The air saturation level must be $\leq 103\%$ after warming.

test observations and measurements (except 1st. paragraph),⁷; and test endpoints and calculations,⁸.

SPECIFIC PROCEDURES FOR TESTING RECEIVING WATER SAMPLES

The sample volume required from each location to be tested, including a suitable upstream location to be used as a control, is 1 litre per day. If necessary, 3 litre samples can be collected every 3 days over a 10 day period.

See Environment Canada EPS Report, 1/RM/28, Section 7, pp. 46-47 for information on sample collection, labelling, transport and storage,⁵; preparing test solutions; control/dilution water; test observations and measurements; and test endpoints and calculations, (see also ⁸, except that a dose/response curve is not produced for receiving water samples).

⁶Three ≈10d old sacfry and three swim-up fry should be used in 96h tests for lethality of all samples, using 100% concentration, without aeration.

⁷See also as described in this protocol, in "Solution renewal", p.16, except that observations of faecal threads and the amount of excess food compared to the controls may only be possible in lower concentration groups if the exposure solution is dark or contains a large amount of solids at the concentration previously estimated to be close to the incipient mortality level.

⁸Biological endpoints are as described in this protocol, in "Post Exposure Procedures", pp.18-19, ie. final weights, (for calculating individual wet weight gains), followed by observations on yolk sac absorption, indicating delayed development, any visible abnormalities including swelling or haemorrhaging, and the shape of the dose-response curve in the highest concentration group(s); an apparent asymptote developing in these groups indicates edema and invalidates the use of their wet weight gains as growth data. This rarely occurs when using sub-incipient mortality levels of toxicants, (see Neville,1995.)

REPORTING REQUIREMENTS See Environment Canada EPS Report, 1/RM/28, Section 8, pp.48-50, for general reporting requirements, and requirements for test substances; test organisms; test facilities and apparatus; control/dilution water; test method; and test conditions; **with the following additions or changes:-**

- the number of samples used and age of sample on each day, for effluents, leachates, elutriates and receiving waters;
- the proportion of fungussed or abnormally developed eyed eggs and sacfry during culturing;
- the number of stressed sacfry after the initial weighing procedure;
- the shape of individual chambers and replicate exposure tanks, and the volume of exposure solution used, if different from those used in developing this protocol, (see description on p. 5). This system was carefully developed to optimise the simplicity and cost effectiveness of the test procedures, as well as the sensitivity and reproducibility, and to minimise loss of volatile chemicals during the exposure period. Changes to this system must take these requirements into consideration, and must be justified and fully described in the report;
- for measured water quality variables, see in this protocol the second paragraph of "Preparing test solutions", (p. 11); and of the section "Control/dilution water" (p. 12), as well as sections 3.4 and 4.3.5 in EPS Report 1/RM/28;
- any changes from the standard conditions used in this protocol: (flowthrough tests and/or aeration should not be used with this protocol since the fish must be exposed and fed individually);
- conditions and procedures for measuring the LOEC and NOEC of reference

toxicants, their respective ICx, and the MSD from the control.

Test results from all groups should include the following:-

- initial wet weights ($\bar{x} \pm \text{s.d.}$ (n) and c.v. (all groups)
 - wet weight gain ($\bar{x} \pm \text{s.d.}$ (n) and c.v. (controls), $\bar{x} \pm \text{s.d.}$ (n) test groups
 - all test groups significantly different from the control
 - the statistical test(s) used
 - the LOEC and ICx, NOEC and ICx for growth reduction, the MSD (% and mg),
 - any accidental or toxicant induced mortalities that occurred, and the length of exposure before death
 - the length of the exposure period if the normal 12 day test had to be prolonged, (see p.22, Specific Procedures For Testing Individual Chemicals)
 - the dose/growth response curve
 - any groups or individual fish that had to be excluded due to edema
 - maximum width of exposed yolk sac surface compared to the controls
- (% of group in each category of severity)
- age at onset of feeding and faecal production
 - estimated amount of food consumption compared to the controls
 - maintained or reduced food consumption in each group by the end of the test
 - any abnormal appearance or behaviour noted during the test
 - summarised mean group oxygen concentrations compared to the controls in pre and post 24h exposure samples, and post 18h exposure during feeding,
 - summaries of the chemical parameters measured in the pre- and post-24h exposure samples on the second and penultimate days of the test compared to the controls or Ontario Provincial Water Quality Objectives for surface waters
 - results of preliminary range finding tests.

REFERENCES

- Abernethy, S.G. and G.F. Westlake. 1989. Guidelines for pH adjustment of effluent samples for toxicity testing. Ontario Ministry of the Environment, Rexdale, Ontario, 11p. [ISBN No. 0-7729-5947-1].
- Environment Canada, 1992. Environmental Protection Series, Biological Test Method: Toxicity tests using early life stages of salmonid fish (rainbow trout, coho salmon, or Atlantic salmon). Report EPS 1/RM/28. [ISBN 0-662-20555-3].
- Hodson, P.V., R. Parisella, B. Blunt, B. Grat, and K.L.E. Kaiser. 1991. Quantitative structure-activity relationships for chronic toxicity of phenol, p-chlorophenol, 2,4-dichlorophenol, pentachlorophenol, p-nitrophenol and 1,2,4-trichlorobenzene to early life stages of rainbow trout (Oncorhynchus mykiss). Can. Tech. Rept. Fish. Aquat. Sci., 1784, 55p.
- Neville, C.M. 1995. Short-term early life stage growth test using sac fry and early swim-up stages of rainbow trout (Oncorhynchus mykiss). Method development and data interpretation, illustrated by exposure to copper, sodium dodecyl sulphate, 2,4,5-trichlorophenol, and 3,4-dichloroaniline. Ontario Ministry of the Environment and Energy Publication, 63p. [ISBN 0-7778-3649-1].



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